ZOOLOGICAL SCIENCE 11: 511–515 (1994)

Stiffness Changes of Holothurian Dermis Induced by Mechanical Vibration

Rie Shibayama, Takakazu Kobayashi, Hiroaki Wada, Hiroko Ushitani, Jun Inoue, Toshimitsu Kawakami and Haruo Sugi[1]

Department of Physiology, School of Medicine, Teikyo University, Itabashi-ku, Tokyo 173, Japan

ABSTRACT—The effect of mechanical vibration on the stiffness of catch connective tissue in the dermis of a sea cucumber *Stichopus japonicus* was studied using the dermis strip preparation, held between a force transducer and a vibrator. During the application of vibration (peak-to-peak amplitude, 2–10%; 2–20 Hz), the stiffness of fresh, stiffened preparations increased by 40–200%, and then stayed constant or slowly decreased. After the stiffness reached a maximum, pause of vibration (5–20 min) had no effect on the steady level of stiffness, except that the stiffness initially showed a higher value on reapplication of vibration. The stiffness of non-fresh, softened preparations showed a much more marked transient increase during the period of vibration. Electrical stimulation either increased or decreased the stiffness by 10–20% in some preparations examined. The vibration-induced stiffness changes were not affected appreciably by Ca^{2+}-free, high-Ca^{2+} (100 mM) and high-K^+ (100 mM) solutions, acetylcholine (10^{-3} M), and low temperatures (1–2°C). These results are discussed in connection with nervous control of the dermis stiffness.

INTRODUCTION

Holothurians (sea cucumbers) are known to stiffen their body wall in response to mechanical stimuli [14, 15, 18]. The stiffening of the body wall results from an increase in stiffness of the dermis, which constitutes most of the thickness of the body wall. The dermis is mainly composed of extracellular materials containing a network of collagen fibres, no muscle cells being present [6]. The dermis of sea cucumbers is therefore called a catch connective tissue, since its stiffened state can be maintained for a long period of time [12]. Motokawa has shown that the stiffness of the dermis can be varied by a number of factors such as acetylcholine, changes in ionic composition of the medium, coelomic fluid, and mechanical and electrical stimulation [3–5, 7–11]. In the above studies, however, the effects of these factors were examined mainly by recording the rate of extension of the preparation under a constant load. As the preparation is being elongated during the course of experiments, it is difficult to study systematically the effect of mechanical stimulation, which is regarded as being a more natural stimulation to the animal.

The present work was undertaken to study the effect of mechanical vibration on the stiffness of the sea cucumber dermis strip preparations, which were held between a force transducer and a vibrator. It was found that mechanical vibrations are effective in changing the stiffness of the preparation.

Accepted June 17, 1994
Received April 4, 1994
[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation

Sea cucumbers *Stichopus japonicus* were collected at the Misaki Marine Biological Station and kept in aerated sea water. The dermis, containing no muscular layers, was cut from the animal, and trimmed to obtain a dermis strip (0.5–1 cm long) with a square cross-section (ca. 1.5×1.5 mm). The dermis strip preparation was mounted horizontally in an experimental chamber (3 ml) between a force transducer (UT-100, Shinko: compliance 1 μm/g, resonant frequency 330 Hz) and a vibrator (model-201, Ling). Both ends of the preparation were firmly glued to the extensions of the force transducer and the vibrator.

The experimental chamber was filled with the standard experimental solution (artificial sea water) which had the following composition (mM): NaCl, 497; KCl, 10; $CaCl_2$, 20; $MgCl_2$, 52 (pH 7.2 by $NaHCO_3$). When the concentrations of K^+ and Ca^{2+} were changed, osmotically equivalent amounts of Na^+ were added or removed. Solutions in the chamber were exchanged from time to time using a water-vacuum suction tube. Experiments were made at room temperature (19–22°C), unless otherwise stated.

Stiffness measurement

The preparation was held at its slack length (L_o), i.e. the length at which the resting force was just barely detectable, and was subjected to continuous sinusoidal vibrations (peak-to-peak amplitude, 2–10% of Lo; 2–20 Hz) with the vibrator driven by a power amplifier to which sinusoidal voltages from a waveform generator (model 164, Wavetek) were fed. The length changes of the preparation were recorded with a light source-photodiode system attached to the shaft of the vibrator. In each experiment, the amplitude of vibration was kept constant, so that the amplitude of the vibration-induced force changes was taken as a measure of the stiffness of the dermis strip preparation. The length and force changes of the preparation were simultaneously recorded with an ink-writing oscillograph, or with a digital oscilloscope (model 310, Niolet) on a fast time

base.

Electrical stimulation

In some experiments, the preparation was placed in contact with 10–18 Pt wire electrodes, which were fixed to the bottom of the experimental chamber and connected as alternate anodes and cathodes, and was stimulated electrically with sinusoidal a.c. currents (20–30 V, 20–50 Hz) from an electronic stimulator (SEN-3301, Nihon Kohden).

Electron microscopy

The dermis strip preparation was fixed with a 2.5% glutaraldehyde solution containing 0.6 M sucrose and 2 mM $CaCl_2$ (pH 7.2 by 0.1 M cacodylate buffer). The tissue was then cut into small pieces, postfixed in 2% OsO_4, dehydrated with a graded series of ethanol, and embedded in Quetol 812. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife, double stained with uranyl acetate and lead citrate, and examined with a JEOL JEM 1200EX electron microscope.

RESULTS

Force changes in response to vibrations

Fig. 1A shows typical force changes of the dermis strip preparation in response to externally applied sinusoidal vibration on a fast time base. Since the preparation was initially kept at its slack length L_o, only the upstroke phase of the applied vibration was effective in stretching the preparation to produce the resulting force changes. It was not possible to produce the force changes taking place symmetrically around a constant level of resting force, because the resting force in the stretched preparation decayed rapidly with time, reflecting the highly extensible nature of the dermis. The force-length loop in response to vibration was narrow and nonsymmetrical in shape with clockwise rotation (Fig. 1B).

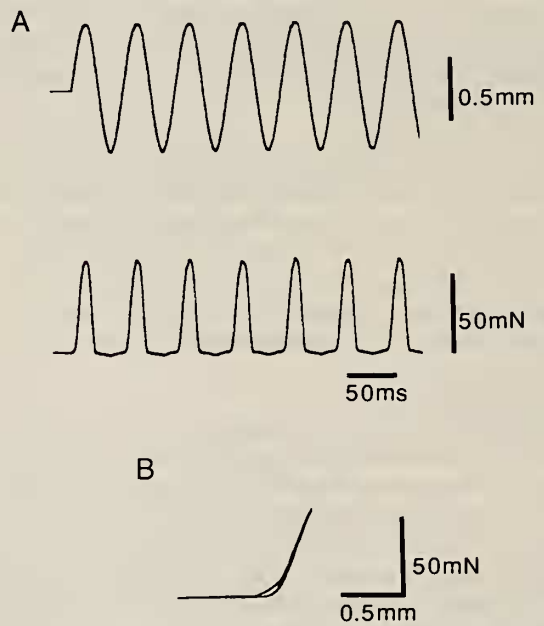

FIG. 1. Force changes of the dermis strip preparation in response to sinusoidal vibrations (5% of L_o, 20 Hz). (A) Length (upper trace) and force (lower trace) changes of the preparation. Note that the preparation is stretched during the upstroke phase of each sinusoidal wave to develop a force. (B) Force-length loop of the preparation.

In the present study, we focused attention on the magnitude of force developed during the stretch phase of vibration of constant amplitude as a measure of the stiffness of the preparation. The time course of the force changes did not change appreciably when the stiffness of the preparation changed markedly with the applied vibrations; in other words, the force changes were scaled according to their amplitude. The above features of the force changes indicate that the amplitude of the vibration-induced force changes serve as a valid measure of the stiffness of the preparation.

Vibration-induced stiffness changes

The sinusoidal vibrations were found to be effective in producing the stiffness changes of the dermis preparations, which were variable depending on the initial state of the preparation. When the dermis preparations were cut from the animal, it always stiffened its body wall. As a result, freshly dissected dermis preparations were obviously much stiffer than the dermis in freely moving animals. On application of vibration, the stiffness of fifteen fresh dermis preparation obtained from ten different animals increased by 40–200% for the first 2–10 min after the beginning of vibration, and then stayed at a constant level (Fig. 2A) or decreased slowly with time (Fig. 2B). The magnitude of the force response at the beginning of vibration (5% of L_o, 20 Hz) ranged from 5 to 20 mN, while the magnitude of force response when the stiffness reached a maximum ranged from 20 to 50 mN.

Fig. 3 shows the effect of pause (5–20 min) of vibration on the stiffness, which had reached a maximum by the

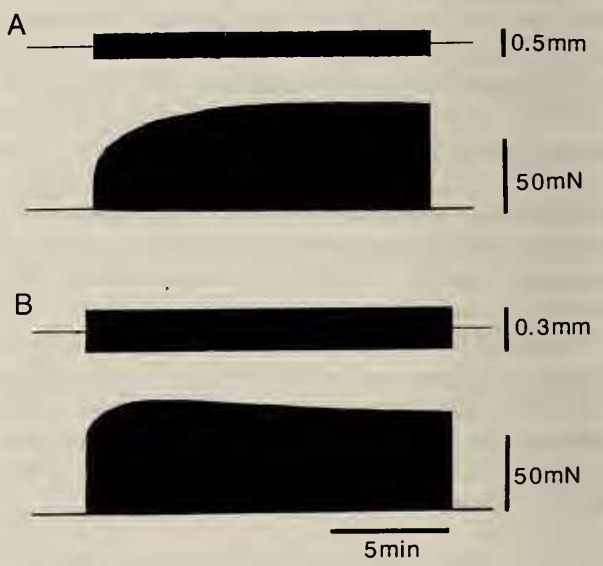

FIG. 2. Records showing the stiffness increase in fresh, stiffened preparations induced by vibration (5%, 20 Hz). The stiffness increased to a maximum value, and then stayed constant (A), or decreased slowly (B).

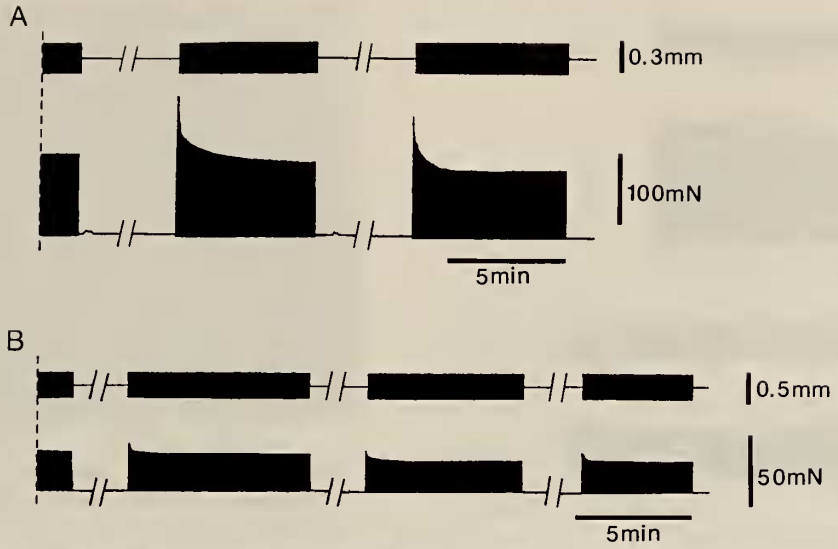

FIG. 3. Records showing the stiffness increase taking place during the period of pause in the preparations, in which the stiffness has reached a maximum by preceding vibration. The degree of the stiffness increase after each pause (10–20 min) of vibration (5%, 20 Hz) is much larger in A than in B. In both A and B, the increased stiffness decreases rapidly to the level equal to that at the end of preceding vibraton.

FIG. 4. Marked transient stiffness increase induced by vibration (2%, 10 Hz) in a nonfresh, softened preparation.

preceding vibration. At the beginning of reapplication of vibration, the stiffness was larger than the value at the end of preceding vibration, but decreased rapidly (in 1 min) to a value nearly as large as that at the end of preceding vibration; then the stiffness stayed at a constant level or decreased slowly with time. The degree of the transient stiffness increase observed after a pause of vibration was variable. In five out of seven preparations examined, the stiffness increased by 70–80% of the steady value at the end of preceding vibration (Fig. 3A), while in the rest two preparations the corresponding stiffness increase was less than 10%.

If, on the other hand, the dermis preparations were kept in the standard experimental solution for more than 10 hr, they tended to become much softer compared with the fresh preparations. Consequently, the length of L_o in the softened preparation was not accurately defined based on the just detectable resting force; instead, L_o was defined as the length at which the preparation became just taut. In such nonfresh, softened preparations, the stiffness exhibited much more marked changes in response to vibrations compared to fresh preparations. As shown in Fig. 4, the stiffness was very small at the beginning of vibration, but increased gradually during the application of vibration to reach a peak, and then decreased slowly with time. In seven softened preparations obtained from five different animals, the magnitude of the force changes when the stiffness was maximum during the application of vibration (5% of L_o, 20 Hz) was 20–30 mN. In three preparations, the above marked transient stiffness increase could be repeated two to three times when vibrations were repeatedly applied after pauses of 20–30 min, though the maximum stiffness value attained during vibration decreased each time of application of vibration.

The above marked effect of vibration on the softened preparations, however, could not be studied in more detail because the softened preparations tended to break with prolonged application of vibration.

Effect of electrical stimulation

To examine the effect of electrical stimulation on the stiffness of the dermis preparation, the fresh preparations were subjected to continuous vibration, and electrical stimulation was applied when the stiffness reached a constant value. In seven out of eleven preparations examined, electrical stimulation had no appreciable effect on the steady level of stiffness. In the rest four preparations, however, the stiffness increased by 10–20% (Fig. 5A) or decreased by 10–20% (Fig. 5B) during the application of electrical stimulation. After the cessation of electrical stimulation, the stiffness

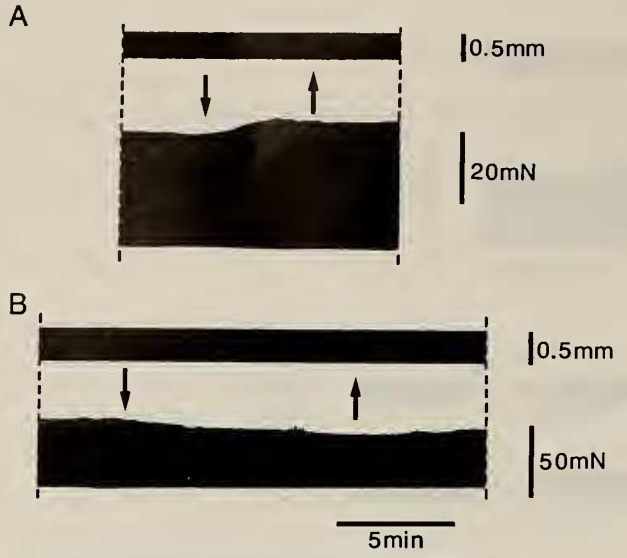

FIG. 5. Effect of electrical stimulation on fresh preparations. Downward and upward arrows indicate onset and cessation of electrical stimulation respectively. In A, the stiffness increased to a higher steady level, while in B the stiffness decreased to a lower value with subsequent slow recovery towards the initial value.

either stayed nearly constant (Fig. 5A) or returned slowly towards the level before stimulation (Fig. 5B)

Effect of ions, acetylcoline and low temperature

Since the rate of extension of the dermis strip preparation is known to be influenced by various ions and drugs such as acetylcholine, we examined the effect of Ca^{2+}-free, high·Ca^{2+} (100 mM), high·K^{+} (100 mM) and acetylcholine (10^{-3} M) solutions on the stiffness of eight fresh preparations, by applying these solutions either during the period of vibration or during the period of pause of 10–30 min. No appreciable effect of these solutions was observed on the subsequent time course of vibration-induced stiffness changes.

As nerve impulse propagation can be blocked by low temperatures (Parker, 1941), we also examined the effect of lowering temperature of the experimental solution to 1–2°C by circulating precooled water around the experimental chamber. In four preparations examined, the time course of vibration-induced stiffness changes was not appreciably affected by low temperatures.

Ultrastructure of the dermis

As shown in Fig. 6, the dermis of *Stichopus japonicus* contained cellular elements consisting of cells with large vacuoles ("vacuole cells") [6], cells without vacuoles ("morula cells") [2], and nerve cells containing clear and cored vesicles. These features are similar to those of the dermis of other species of sea cucumbers, *Thyone bareus* [2] and *Stichopus chloronotus* [6].

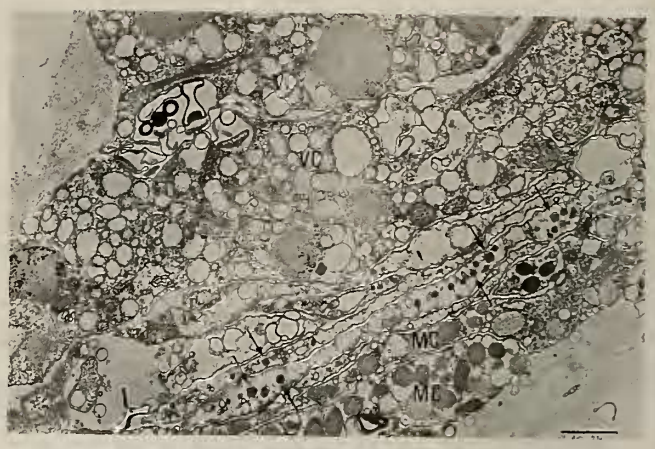

FIG. 6. Electron micrograph of cellular elements in the dermis tissue. Nerve cells containing vesicles (arrows) are closely apposed to vacuole cells (VC) and morula cells (MC). Scale bar, 1 μm.

DISCUSSION

The present experiments have shown that externally applied vibration is effective in changing the stiffness of the dermis preparation, as measured by the amplitude of the vibration-induced force changes. Evidence has been accumulating that the dermis stiffness is controlled by two different types of nerve, one stiffening and the other softening the dermis [12]. Concerning the stiffening of the dermis, Matsuno & Motokawa [1] recently demonstrated that the stiffening of the dermis is associated with release of calcium ions from the lumen of the vacuole cell into the dermis connective tissue, using the pyroantimonate method that is effective in studying calcium translocation associated with the contraction-relaxation cycle of various muscle cells [16, 17]. The present results will be discussed below on the above basis.

In the present study, vibration was used both to induce the stiffness changes and to measure the stiffness (Fig. 1). In fresh, stiffened preparations, the stiffness increased to a maximum for the first 2–10 min after the beginning of vibration (Fig. 2). The applied vibration may first mechanically stimulate the nervous elements in the dermis, which in turn causes release of "dermis-stiffening" substance (possibly calcium ions) from the vacuole cells closely apposed to the nervous elements (Fig. 6) to increase the dermis stiffness. On this basis, the wide range of the extent of vibration-induced stiffness increase (40–200%) depends on the variation in the degree of stiffening of the dermis before the application of vibration, as the nerves in the dermis are mechanically stimulated when the dermis preparation is being prepared. Once the stiffness reached a maximum by the applied vibration, it stayed constant or decreased only very slowly, indicating that vibration was no longer effective in changing the stiffness. This view is supported by the result that pauses of vibration had no appreciable effect on the stiffness; after each pause the stiffness first showed a higher value than that during the preceding vibration, but then

decreased rapidly to a value as large as that during the preceding vibration (Fig. 3). This indicates that the apparent stiffness increase taking place during the period of pause is rapidly eliminated by the subsequent vibration. Our impression at present is that the above rapid stiffness decrease at the beginning of reapplication of vibration might result from a thixotropic nature of the stiffened dermis rather than the action of "softening" nerve; it may be that cross-linkages between the molecules constituting the dermis tissue are partly broken by vibration to result in the initial rapid stiffness decrease. The slow stiffness decrease might also be explained in terms of slow breaking of the cross-linkages. Of course, the possibility is not excluded that the stiffness decrease is due to the action of "softening" nerves.

On the other hand, nonfresh, softened preparations exhibited a marked transient stiffness increase in response to vibration (Fig. 4). In such preparations, "dermis-softening" substance would be expected to be released from the vacuole cells into the dermis tissue as the preparation is kept standing over many hours, while "dermis-stiffening" substance might be taken up into the vacuole cells. The application of vibration might then release "dermis-stiffening" substance, which may overcome the existing effect of "dermis-softening" substance to cause a marked stiffness increase. The transient nature of the above stiffness increase might at least in part, be because the effect of "dermis-stiffening" substance is not long-lasting in the presence of "dermis-softening" substance.

In the present experiments, the initial application of vibration caused only an increase of the dermis stiffness (Figs. 2 and 4). This may be related to the fact that the dermis of living animals can be readily stiffened even by a brief weak mechanical stimulation, while its softening requires intense repeated mechanical stimulation [12]; the applied vibrations were probably insufficient in intensity to cause softening of the preparation. The result that electrical stimulation was effective in changing the stiffness only in some preparations (Fig. 5) may be because externally applied current can not effectively stimulate the nervous elements that are only sparsely distributed in the dermis tissue.

Concerning the mechanism of vibration-induced release of "stiffening" and "softening" substances, the only information obtained in the present study is that propagation of action potential may not be involved, since the vibration-induced stiffness changes were not appreciably influenced by low temperatures that block nerve impulse condition. Though the rate of extension of the dermis strip preparation is affected by Ca^{2+}-free, high-Ca^{2+} and high-K^{+}-solutions and acetylcholine [12], these factors had no appreciable effect on the vibration-induced stiffness changes. It seems possible that diffusion of substances in the dermis tissue occurs much more readily if the preparations is extended many times the initial length. Another possibility may be that these factors affect the "viscosity" of the preparation as measured by its rate of extension with a constant load, but not "stiffness" as measured by the amplitude of vibration-induced force changes in the preparation held at L_o.

REFERENCES

1 Matsuno A, Motokawa T (1992) Evidence for calcium translocation in catch connective tissue of the sea cucumber *Stichopus chloronotus*. Cell Tissue Res 267: 307–312

2 Menton DN, Eisen AZ (1970) The structure of the integument of the sea cucumber, *Thyone briareus*. J Morphol 131: 17–36

3 Motokawa T (1981) The stiffness change of the holothurian dermis caused by chemical and electrical stimulation. Comp Biochem Physiol 70C: 41–48

4 Motokawa T (1982*a*) Factors regulating the mechanical properties of holothurian dermis. J exp Biol 99: 29–41

5 Motokawa T (1982*b*) Rapid change in mechanical properties of echinoderm connective tissues caused by coelomic fluid. Comp Biochem Physiol 73C:223–229.

6 Motokawa T (1982*c*) Fine structure of the dermis of the body wall of the sea cucumber, *Stichopus chloronotus*, a connective tissue which changes its mechanical properties. Galaxea 1: 55–64

7 Motokawa T (1984*a*) The viscosity change of the body-wall dermis of the sea cucumber *Stichopus japonicus* caused by mechanical and cheical stimulation. Comp Biochem Physiol 77A: 419–423

8 Motokawa T (1984*b*) Viscosity increase of holothurian body wall in response to photic stimulation. Comp Biochem Physiol 79A: 501–503

9 Motokawa T (1986) Effects of ionic environment on viscosity of catch connective tissue in holothurian body wall. J exp Biol 125: 71–84

10 Motokawa T (1987*a*) Cholinergic control of the mechanical properties of the catch connective tissue in the holothurian body wall. Comp Biochem Physiol 86C: 333–337

11 Motokawa T (1987*b*) Calcium dependence of viscosity change caused by cations in holothurian catch connective tissue. Comp Biochem Physiol 87A: 579–582

12 Motokawa T (1987*c*) Catch connective tissue: A key character for echinoderms' success. In "Echinoderm Biology" Ed by RD Burke, PV Mladenov, P Lambert , RL Parsley, AA Balkema, Lotterdam, pp 39–54

13 Parker GH (1941) Melanophore bands and areas due to nerve cutting, in relation to the protracted activity of nerves. J gen Physiol 24: 483–504

14 Scott RSH, Hepburn HR, Joffe I, Heffron JJA (1974) Mechanical defensive mechanism of a sea cucumber. S Afr J Sci 70: 46–48

15 Serra-von Buddenbrock E (1963) Études physiologique et histologique sur le tégument des holothuries (*Holothuria tubulosa*). Vie Milieu 14: 55–70

16 Sugi H, Suzuki S, Daimon T (1982) Intracellular calcium translocation during contraction in vertebrate and invertebrate smooth muscles by the pyroantimonate method. Canad J Physiol Pharmacol 60: 576–587

17 Suzuki S, Sugi H (1989) Evaluation of the pyroantimonate method for detecting intracelular calcium translocation in smooth msucle fibers by the X-ray microanalysis of cryosections. Histochemistry 92: 95–101

18 Uexküll J von (1926) Die Sperrmuskulatur der Holothurien. Pflüg Arch ges Physiol 212: 1–14